FEBS Letters 580 (2006) 1771–1774

Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes

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Received 9 January 2006; revised 12 February 2006; accepted 13 February 2006

Available online 24 February 2006

Edited by Barry Halliwell

Abstract **Sulforaphane (SFN) is an activator of the transcription factor Nrf2, which plays a critical role in metabolism and excretion of xenobiotics. Exposure of primary mouse hepatocytes to SFN resulted in activation of Nrf2 and significant elevation of protein expressions responsible for excretion of arsenic into extracellular space. Pretreatment with SFN 24 h prior to arsenite exposure reduced not only arsenic accumulation in the cells but also cellular toxicity of this metalloid. Therefore, our findings indicate a potential function of SFN in reducing cellular arsenic levels, thereby diminishing arsenic toxicity.**

Keywords: Sulforaphane; Nrf2; Glutathione; Arsenic; Chemoprevention

1. Introduction

Inorganic arsenic is a metalloid that is ubiquitously distributed in the earth's crust. High levels of arsenic contamination of groundwater in an endemic area of chronic arsenic poisoning in east Asia have led to severe environmental problems. It is well recognized that in humans, inorganic arsenic undergoes reduction and oxidative methylation and thus monomethyl and dimethyl metabolites of arsenic are excreted into urine [1,2]. Alternatively, inorganic arsenite is bound to glutathione (GSH), which is synthesized by γ-glutamylcysteine synthase (γ-GCS), by chemical and/or enzymatic processes through presumably GSH *S*-transferases (GSTs) to yield arsenic triglutathione ($As(GS)_3$) [3,4]. This polar metabolite is excreted into extracellular space via multidrug resistance-associated protein (MRP) [4,5]. Interestingly, Hayakawa et al. [6] reported recently that the GSH adduct of arsenic is an intermediate for methylarsenic, suggesting that the GSH conjugation is essential for facilitation of arsenic excretion. Because cytotoxicity of arsenic is thought to be associated with its accumulation in cells, it is postulated that a possible approach to chronic arsenic poisoning is activation of transcription factors that control proteins such as γ-GCS, GSTs and transporters associated with arsenic excretion into extracellular space.

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Abbreviations: SFN, sulforaphane; Nrf2, nuclear factor E2 related factor 2; γ-GCS, γ-glutamylcysteine synthase; γ-GCS_H, γ-GCS heavy subunit; γ-GCS_L, γ-GCS light subunit; GSH, glutathione; GST, GSH *S*-transferase; MRP, multidrug resistance-associated protein

Nuclear factor E2 related factor 2 (Nrf2) has been identified as a transcription factor regulating proteins that participate in metabolism and excretion of organic chemicals [7,8]. For this reason, treatment with chemicals activating Nrf2 before exposure to carcinogens has been attempted [8,9]. For example, we found that pretreatment with an Nrf2 activator, oltipraz, reduced tumor incidence caused by alkyl nitrosoamine by inducing phase II enzymes such as UDP-glucuronosyltransferase, thereby decreasing tissue levels of the reactive metabolite of *N*-nitrosobutyl(3-carboxypropyl)amine [10]. Sulforaphane (SFN), a compound found in broccoli sprouts and broccoli, is also known to be a potent Nrf2 activator and is capable of preventing toxicity of organic chemicals [9,11]. To our knowledge, however, the protective effect of Nrf2 activators on arsenic-mediated cytotoxicity has not been reported. Our rationale was that upregulation of proteins responsible for excretion of arsenic into extracellular space could lead to substantial reduction of steady-state levels of arsenic in cells, thereby diminishing cellular toxicity caused by arsenic exposure. Here, we report, for the first time, that SFN is an effective agent in reducing cellular arsenic levels in primary mouse hepatocytes and thus the cytotoxicity of arsenic.

2. Materials and methods

2.1. Materials

Sodium arsenite (iAs(III)) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); SFN from LKT Laboratories (St. Paul, MN, USA); anti-MRP1 from Alexis Biochemicals (San Diego, CA, USA); anti-Nrf2, anti-γ-GCS_H, and anti-γ-GCS_L from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-GSTA1, anti-GSTM1, and anti-GSTP1 from Oxford Biomedical Research (Oxford, MI, USA); anti-actin from Sigma (St. Louis, MO, USA); and anti-heme oxygenase-1 (HO-1) from Stressgen (Victoria, Canada). Anti-GSTA4 and anti-arsenite methyltransferase (CYT19) were kindly provided by Dr. Akira Hiratsuka (Tokyo University of Pharmacy and Life Science, Japan) and by Dr. Yayoi Kobayashi (National Institute for Environmental Studies, Japan), respectively. All other reagents and chemicals used were of the highest grade available.

2.2. Isolation and culture of primary mouse hepatocytes

Primary hepatocytes were isolated from 6- to 9-week-old C57BL/6J male mice by collagenase perfusion according to the method described by Liang et al. [12] with slight modification. Briefly, the liver parenchymal hepatocytes were seeded at a density of 8×10^4 cells/cm^2 with William's medium E containing 20 mM L-alanyl-L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin, 0.1 μM dexamethasone, and supplemented with 10% FBS. They were plated into culture plates coated with pig type I collagen (IWAKI, Japan). The cultures

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.031

were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. We stimulated cells with SFN from the 3rd day of isolation to stabilize cell response because transient HO-1 expression was observed just after isolation of the hepatocytes. All experiments were performed at least twice using different cell culture preparations.

2.3. Assay of cytotoxicity

MTT assay was performed as previously described [13].

2.4. Alteration in protein levels

Western blotting analysis was performed as previously described [14]. Nuclear fraction was prepared by using nuclear extraction reagent (PIERCE, IL, USA) according to the manufacturer's protocol. Crude membrane fraction was prepared by differential centrifugation as described by Germann et al. [15].

2.5. Measurement of intracellular GSH

Cellular GSH content was determined according to the method described by Vignaud et al. [16] with slight modification (HPLC-ECD). Briefly, 10 μl of samples obtained from the primary mouse hepatocytes were loaded onto a C18 Beckman ODS column (250 mm × 4.6 mm, i.d.) equipped with a guard column (17 mm × 4.6 mm, i.d.).

2.6. Measurement of arsenic concentrations

Cellular arsenic was measured by ICP-MS (HP4500 plus, Yokokawa Analytical Systems, Tokyo, Japan) after digestion of those samples with nitric acid–H_2O_2 as previously described [17]. Cells were plated into 12-well culture plates.

3. Results

It has been reported that chemicals which block GSH synthesis and transporters for arsenic excretion into extracellular space cause acceleration of arsenic accumulation in cells and thus accelerate cytotoxicity caused by arsenic [18]. Exposure of primary mouse hepatocytes to iAs(III) resulted in a concentration-dependent cytotoxicity with a LD_{50} value of 30 μM at 48 h after arsenic exposure. Pretreatments with buthionine sulfoximine (BSO, γ-GCS inhibitor), with ethacrynic acid (EA, GST inhibitor), with MK-571 (MRP antagonist) caused significant enhancement of iAs(III)-mediated cytotoxicity, respectively (Fig. 1), supporting previous reports that arsenic accumulation in hepatocytes is associated with increased hepatotoxicity. MTT assay showed that the viability of the cells increased when the concentration of MK-571 was elevated. Such an observation was in agreement with Vellonen et al.'s report [19] that MK-571 interferes with MTT assay in cells, leading to underestimation of cellular toxicity caused by chemicals.

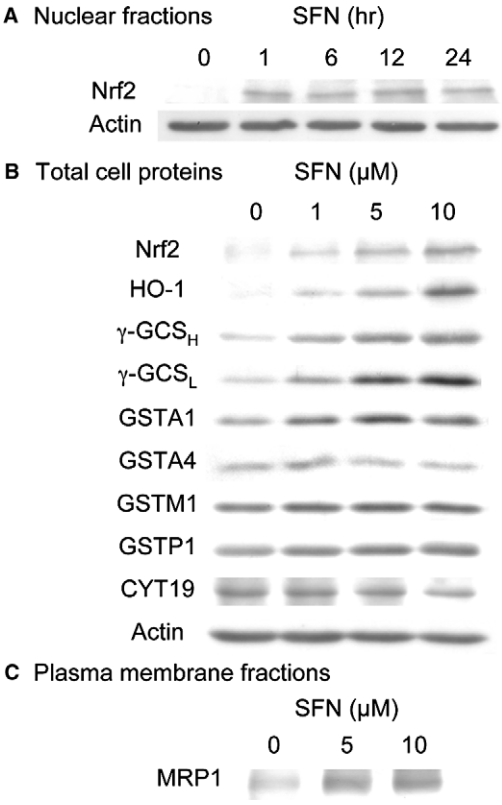

Fig. 2. SFN activates Nrf2 in primary mouse hepatocytes. (A) Western blot analysis to detect nuclear fraction of Nrf2 after the treatment of cells with 10 μM SFN for 0, 1, 6, 12, and 24 h. Actin was used as an internal control. (B) Western blot analysis of Nrf2, HO-1, γ-GCS_H, γ-GCS_L, GSTA1, GSTA4, GSTM1, GSTP1, and CYT19 in whole cell lysates. Cells were treated with 0–10 μM SFN for 24 h. Actin was used as an internal control. (C) Western blot analysis of MRP1. Cells were treated with 0–10 μM SFN for 24 h. Crude membrane fractions were used.

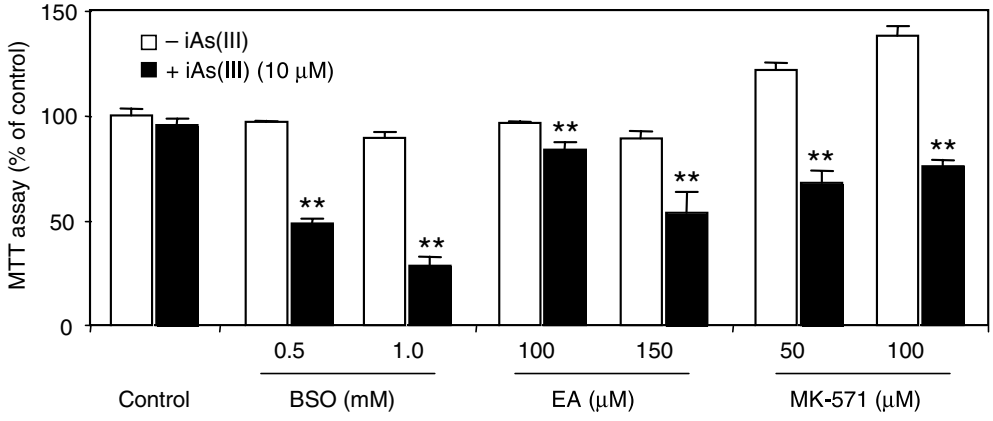

Fig. 1. The effects of BSO (γ-GCS inhibitor), EA (GST inhibitor), and MK-571 (MRP antagonist) on arsenic-induced cytotoxicity in primary mouse hepatocytes. Cells were exposed to iAs(III) (10 μM) for 48 h in the absence or presence of BSO (0.5 or 1.0 mM), EA (100 or 150 μM), or MK-571 (50 or 100 μM). BSO, EA, and MK-571 were respectively added to the culture media 6 h (BSO) or 1 h (EA and MK-571) prior to the addition of iAs(III). Each value represents the means ± S.D. of four determinations. **, $P < 0.01$.

SFN (1–10 μM) activated Nrf2 as evaluated by an increase of protein expression in total hepatocyte protein lysate and a translocation to the nucleus (Fig. 2). Under these conditions, among the proteins regulated by Nrf2, HO-1, GSTA1, γ-GCS heavy subunit (γ-GCS_H), γ-GCS light subunit (γ-GCS_L), and MRP1 were upregulated by exposure to SFN, whereas few appreciable changes in GSTA4, GSTM1, and GSTP1 levels were seen. Note that expression of CYT19 catalyzing oxidative methylation of iAs(III) [20] was not affected by treatment with SFN.

If γ-GCS subunits are induced by SFN, intracellular GSH concentration should be elevated because this protein is known to be a rate-limiting enzyme for GSH synthesis. While GSH concentration in primary mouse hepatocytes without SFN exposure was 93.5 ± 2.9 nmol/mg protein ($n = 3$), cellular GSH levels increased by approximately 1.56-fold 24 h after treatment with 5 μM SFN (Fig. 3).

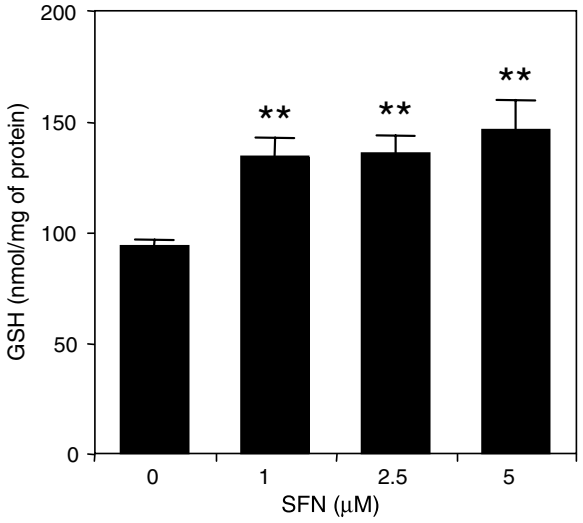

Fig. 3. Enhancement of intracellular GSH by SFN in primary mouse hepatocytes. Cells were incubated with the indicated concentrations of SFN for 24 h. Each value represents the means ± S.D. of three determinations. **, $P < 0.01$.

Because SFN was capable of elevating proteins such as γ-GCS subunits, GST isozymes, and MRP1, all of which are responsible for facilitation of iAs(III) excretion extracellularly, we investigated the effects of SFN on steady-state levels of arsenic and on iAs(III)-mediated cytotoxicity. Pretreatment with SFN (5 μM) for 24 h prior to 5 μM iAs(III) exposure resulted in a significant reduction of cellular accumulation of arsenic (66.8% of control) (Fig. 4A). These results suggest that SFN could inhibit iAs(III)-mediated cytotoxicity through decreased arsenic levels in hepatocytes. Consistent with this notion, SFN had a significant protective effect on arsenic cytotoxicity (Fig. 4B).

4. Discussion

It has been shown that as a chemopreventive agent, SFN is capable of diminishing the cytotoxicity of organic chemicals [11] such as menadione, *tert*-butyl hydroperoxide, and 4-hydroxynonenal. This function is thought to be due to activation of Nrf2, leading to transcriptional induction of drug-metabolizing enzymes and of MRP1 [21,22], which are associated with detoxification of organic chemicals. For example, an active metabolite of aflatoxin B1 is conjugated with GSH by GST isoforms and then translocated to extracellular space through ABC transporters, such as the MRP family [23]. Although these reports showed that SFN decreases cytotoxicity induced by organic chemicals, whether SFN affects cytotoxicity induced by inorganic chemicals remained to be elucidated. In the case of arsenic, it has been reported that iAs(III) readily undergoes GSH conjugation to yield $As(SG)_3$, which is pumped out to extracellular space through MRP1 [4]. Once the GSH adduct of arsenic is formed, cellular GSH should be consumed. Thus, we postulated that increased protein levels of γ-GCS, GST isoforms, and MRP1 during pretreatment with a Nrf2 activator such as SFN would facilitate decreased arsenic accumulation in primary mouse hepatocytes, leading to substantial reduction of cytotoxicity. Consistent with this notion, blockage of GSH biosynthesis, GST and MRP transporters by BSO,

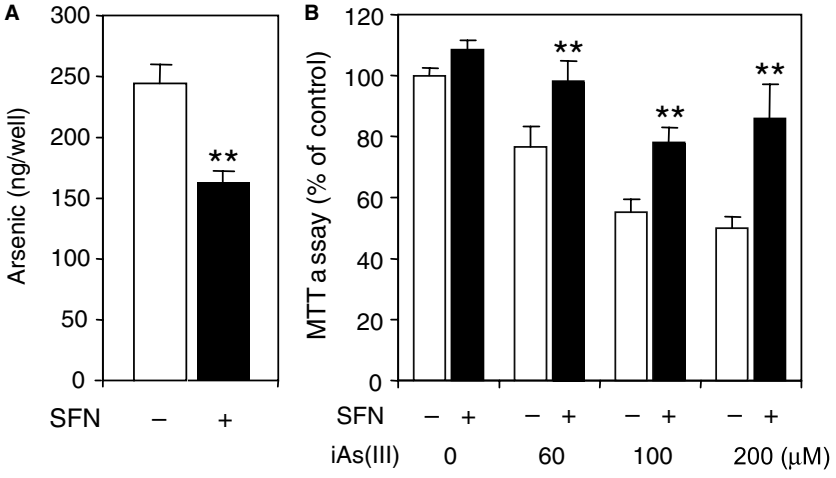

Fig. 4. Effect of SFN on cellular arsenic accumulation and cytotoxicity of iAs(III) in primary mouse hepatocytes. (A) Cells were exposed with either DMSO (0.1%) or SFN (5 μM) for 24 h prior to exposure with 5 μM iAs(III) for 24 h. Each value represents the means ± S.D. of three determinations. (B) Cells were exposed with either DMSO (0.1%) or SFN (5 μM) for 24 h prior to the indicated concentrations of iAs(III). Data are shown as percentages of the control value. Each value represents the means ± S.D. of six determinations. **, $P < 0.01$.

EA, or MK-571 accelerated iAs(III)-mediated cytotoxicity, whereas upregulation of these proteins by pretreatment with SFN suppressed such a toxic action. These results suggest that Nrf2 activators are efficient in diminishing the cellular toxicity of iAs(III) through transcriptional induction of proteins responsible for its GSH conjugation and excretion into extracellular space. However, it should be noted that alternative functions of SFN (e.g., inhibition of iAs(III) uptake) and abolishment of the SFN effect on primary hepatocytes from Nrf2-deficient mice remain to be elucidated.

We reported previously that arsenic is capable of activating Nrf2, thereby upregulating antioxidant proteins (heme oxygenase-I, peroxiredoxin I and A170) and phase II proteins (γ-GCS, NQO1) [24,25]. Also, Liu et al. showed that arsenic induced GST and MRP expressions [18]. These reports suggest that by increasing levels of proteins responsible for excretion of arsenic into extracellular space, arsenic-mediated Nrf2 activation is one protective response against arsenic. To assess the capacity of SFN to act as a chemopreventive agent for arsenic-induced cytotoxicity, we attempted in this study to use this Nrf2 activator instead of arsenic itself. Our study suggests that SFN is a naturally occurring chemical that is able to diminish cellular arsenic levels and thus reduce its cytotoxicity.

Acknowledgements: We thank Drs. Ken Itoh and Masayuki Yamamoto, Doctoral Programs in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, for their earnest encouragement of our study. We are especially grateful to Ms. Flaminia Miyamasu for grammatical correction in the preparation of this manuscript. This work was supported in part by funding from Sumitomo foundation, and by a Grant-in-Aid (#15406004 to Y.K.) for scientific research from the Ministry of Education, Science and Culture of Japan.

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